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The protective effects of astaxanthin against cisplatin-induced retinal toxicity

Running title: Astaxanthin against retinal toxicity

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Summary

This study investigated the toxic effects of an antineoplastic agent, Cisplatin (CIS), on retinal cells and the potential capacity of Astaxanthin (ASTA) to elicit a future therapeutic protocol in CIS-induced retinal toxicity. Six groups were formed for the assessment; control (healthy; Group 1), olive oil (olive oil only; Group 2), ASTA control group (ASTA only, Group 3), the single intraperitoneal (IP) dose of 16 mg/kg CIS (CIS only group; Group 4), 16 mg/kg CIS + 25 mg/kg (IP) ASTA (Group 5) and 16 mg/kg CIS + 75 mg/kg (IP) ASTA (Group 6). On the third day after cisplatin administration, rats in all groups were sacrificed under anesthesia and the analysis of the biochemical parameters and histopathological levels were performed. A significant decrease in GSH levels and increases in MDA, eNOS, 8-OHdG expressions were recorded. Additionally, CIS treatment had caused acidophilic staining in retinal histological appearance. ASTA treatment reduced the increases in MDA, eNOS, and 8-OHdG levels following CIS administration and increased the levels of GSH expressions, as well. These results may suggest the ASTA molecule as a promising option to prevent retinal toxicity in patients receiving CIS treatment for malignant tumors.

Keywords: Astaxanthin, chemotherapy, cisplatin toxicity, eNOS, oxidative stress, retina

Introduction

As an increasing health problem, cancer is a disease which causes multi-organ dysfunction and a major cause of death worldwide [1]. Several researchers point out the ascending pattern of numbers that may possibly affect the population in future and the results are devastating[2, 3]. Moreover, not only the disease itself, also the side effects of the therapy options are another issue to deal with through the way to recovery [4, 5].

Since its first approval as an antineoplastic in 1978, CIS is widely used to treat various cancer types such as head, neck, ovarian, testicular, breast and small cell lung [6]. Its side effects overhearing, kidneys, liver, and central nervous system were widely described in previous studies[7–10]. However, as a rare condition, its retinal toxicity is stated in a few case reports and the mechanism to explain this toxicity is yet to be cleared [11–13].

Oxidation and redox balance in accordance with a variety of enzymes is a vital feature of a human organism[14]. Reduced glutathione (GSH) which is a key element in this cycle has been described as an important molecule to follow up this balance [15]. A failure of antioxidant mechanisms leading to accumulation of reactive oxygen species is suggested to cause an activation of the proapoptotic process which is responsible for cell death and tissue damage [16]. Moreover, studies reporting the positive effect of antioxidant molecules such as selenium, vitamin E and fish oil against these toxicities, support this suggestion in explaining the probable mechanisms [17–19]. Mitochondrial and nuclear DNAs induced by reactive oxygen species show 8-hydroxy-2p-deoxyguanosine (8-OHdG) activation. Although the other components of the DNA interact with ROS as well, thanks to its easy forming pattern, 8-OHdG is the most common biomarker to observe the oxidative damage in cell structures [20, 21]. Malondialdehyde (MDA) is also known as a natural marker of lipid peroxidation and provides proof to determine the severity of tissue damage due to ROS [22, 23].

Astaxanthin (ASTA) is a member of carotenoids family and has been widely used in food industry as a pigment[24, 25]. The first description of ASTA was by Kuhn and Soerensen in 1937 and its effect on antioxidation processes was previously described in several studies in vivo and in vitro, as well [26–29]. Its potential antioxidative and neuroprotective effect was suggested as a therapy option for diabetes, cancer, and hepatotoxicity [30–33]. It is also used as a supplementary medication for dry type-age related macular degeneration patients[34].

This study aimed to assess the toxic effects of CIS on retinal cells and the protective effect of ASTA over this toxicity by investigating biochemical parameters and histopathological specimens. Even though there are few studies focused to discover molecules to reverse this toxicity, to the best of our knowledge, none of the previous studies investigated the effect of ASTA on this toxicity and neither described the histopathological damage zones caused by CIS.

Materials and Methods

Forty-eight male Sprague Dawley rats at the age of 13–15 weeks, and weighing 264.83±7.39 g obtained from the Recep Tayyip Erdogan University Animal Care and Research Unit (Rize, Turkey) were used in this study. All rats received care according to the criteria outlined in the 'Guide for the Care and Use of Laboratory Animals' prepared by the National Academy of Sciences and published by the National Institutes of Health. Recep Tayyip Erdogan University (Rize, Turkey), Animal Ethical Committee approved this study.

Study Design

Animals were kept in Recep Tayyip Erdogan University Animal Care and Research Unit (Rize, Turkey). All animals were maintained and fed in the sterile experimental animal unit environment, having 55-60% humidity, the temperature of 22 ± 3 ° C, and 12-h light:12-h light-dark cycle. Rats were allowed access to commercially available standard rat chow (Bayramoğlu Feed and Flour Industry Trading Corporation Erzurum, Turkey) and tap water ad libitum along the experiment. After sufficient time to comply with the laboratory conditions, 48 experimental animals were divided randomly into six groups.

Group 1 was the control group (n=8). No drugs were injected except for anesthetics. Group 2 acted as the olive oil control group (n=8). Olive oil was used for dissolving ASTA in groups 3-6. This group received intra-peritoneal olive oil only, for 8 days [35, 36]. Group 3 was the ASTA control group (n=8). This group received only intra-peritoneal ASTA 75mg/kg, dissolved in olive oil [37]. Group 4 acted as the CIS only group (n=8). 16mg/ kg single dose CIS (Cisplatin DBL 100mg / 100ml vial, Orna Ilac, İstanbul) was given IP on the 5th day [38]. Group 5 was formed by the 16 mg/kg CIS & 25mg/kg ASTA group (n=8). 25 mg/kg astaxanthin IP was given daily for 8 days, and 16 mg/kg single dose CIS was injected IP on the 5th day. Group 6 was determined to be the 16 mg/kg CIS & 75 mg/kg ASTA group (n=8). 75 mg/kg ASTA IP was given daily for eight days and 16 mg/kg single dose CIS was given IP on the 5th day (Figure 1). Rats in all groups were sacrificed under anesthesia on the third day after CIS administration. Anesthesia was performed to all groups with 50 mg/kg intraperitoneal Ketamine hydrochloride (Ketalar ®, Eczacibası Parke-Davis, Istanbul, Turkey) and 10 mg/kg intraperitoneal Xylazine HCl (Alfazyne ®, Alfasan International BV Woerden, Holland)[39].

Biochemical analysis

Glutathione (GSH) analysis

Eye tissues were prepared with Phosphate buffered saline (PBS; pH: 7.4). The samples were weighed and washed in ice-cold PBS. The GSH assessment was also performed according to the Beutler method[40, 41].

Malondialdehyde (MDA) analysis

The eye-tissue extract incubation was carried out with 900 µL working tubes and 600 µL of TCA solution was added and centrifuged at 3000 rpm for 10 minutes. MDA assessment was performed according to the Dormandy method [42].

Histopathological analysis procedure

The eye tissue specimens were fixed in 10% formalin (Merck, Darmstadt, Germany) for 36 hours and then paraffin embedded by routine histological follow-up protocol procedures. Eye tissue samples were divided into 3-4 µm thick sections by the microtome and sections were stained with Harris hematoxylin and eosin G (H & E, (Merck, Darmstadt, Germany). Histopathological analysis was evaluated under a light microscope (Olympus BX51, Japan) with an Olympus DP20 (Olympus Corp, Japan) camera by two blind histopathologic examinations.

Immunohistochemistry (IHC) analysis procedure

2-3 µm thick eye tissue sections were incubated with Anti-8 Hydroxyguanosine antibody (8 OH-dG, 1:200, Mouse monoclonal, Abcam, UK) and Anti-e-NOS antibody (1:100, Rabbit polyclonal, Abcam, UK). Then, eye tissue sections were incubated with a secondary antibody (Rabbit Specific HRP/DAB [ABC] Detection IHC kit, Abcam, UK). Thereafter, sections were incubated in anti-digoxigenin-peroxidase and were incubated with 0.06% 3, 3-

diaminobenzidine tetrahydrochloride (Sigma Chemical, St. Louis, MO). Eventually, eye sections were counterstained with Harris hematoxylin (Merck, Darmstadt, Germany).

Semi-quantitative analysis

The analysis was evaluated under x40 magnification under a light microscope (Olympus BX51, Japan) by two blinded histopathologists (LT with 10 years of experience and TM eight years of experience). Each IHC stained sections were randomly selected from 20 random areas for semi-quantitative analysis (Table 1)[35].

Quantitative analysis

The retinal thickness (μm) measurement for each eye tissue section in 20 different selected areas was performed with the Olympus DP2-BSW (Olympus Corporation, Tokyo, Japan) software system by two blinded histopathologists.

Statistical analysis

Data were analyzed using the SPSS Statistics 18.0 software (IBM Inc., Chicago, USA). Parametric variables were expressed as a mean ± standard deviation. Parametric variables were tested by using the one-way analysis of variance (ANOVA) followed by the Tukey HSD test. Nonparametric the pars optical retinae thickness and semi-quantitative analysis variables were expressed as the median ± standard deviation, and the differences between the groups were tested by using the Kruskal Wallis test; p values <0.05 were regarded as significant.

Results

Biochemical analysis

The mean MDA levels in the control group were 18.13±1.57 mmol/L, rising to 24.01±1.23 mmol/L in the CIS group (*P*=0.000; Table 2). ASTA 25 mg treatment reduced MDA levels after administration of CIS alone from 24.01±1.23 mmol/L to 16.15±0.66 mmol/L (*P*=

0.000; Figure 2; Table 2). ASTA 75 mg treatment reduced MDA levels after administration of CIS single dose from 24.01±1.23 mmol/L to 15.93±0.22 mmol/L (*P*=0.000; Table 2).

The mean GSH level in the control group was 24.40±1.06 mmol/L and was reduced to 19.19±1.11 mmol/L in the CIS group (*P*=0.000; Table 2). ASTA 25 mg treatment raised mean GSH level from 19.19±1.11 mmol/L to 26.35±1.30 after administration of CIS alone (P=0.000; Table 2). ASTA 75 mg treatment raised mean GSH level from 19.19±1.11 mmol/L to 26.09±1.38 after administration of Cis alone (*P*=0.000; Table 2).

Histopathological analysis

The normal histopathological characteristics of pars optica retinae were demonstrated (Figures 1A-B). Olive oil and only ASTA 75 mg group histopathological structures were similar to the control group (Figures 1C-E). Retinal cells in H&E stained sections from only CIS treatment group had more acidophilic stained histological appearance than the control group, but no sign of a pathology was observed (Figures 1F). In addition, retinal cells in ASTA 25 and 75 treatment groups, no sign of a pathology was observed.

Semi-quantitative analysis

We observed a larger number 8-OHdG positive staining particularly at the horizontal cells and other retinal neurons in the CIS group compared to the control group (p=0.014; Figure 2; Table 3). The ASTA 25 mg and ASTA 75 mg treated groups demonstrated less staining with 8-OHdG especially in horizontal cells and neurons than the CIS only group (*P*=0.014; *P*=0.01; respectively; Figure 2; Table 3).

A larger number of eNOS antibody staining was present in the retinal blood vessels in the CIS only group (*P*=0.025; Figure 3; Table 3). The ASTA 25 mg and ASTA 75 mg treatment decreased eNOS antibody staining positivity in the retinal blood vessels compared to cisplatin only group (p=0.025; Figure 3; Table 3).

Quantitative analysis

The pars optica retinae thickness measurements yielded significant differences in the CIS only group compared to the control group (*P*=0.000)(Table 4). On the other hand, ASTA 25 and 75 mg treatment reduced pars optica retinae thickness after administration of CIS (*P*=0.016, p=0.03; respectively, Table 4).

Discussion

In this animal study model, single dose IP CIS was administered to observe the toxic effects both histopathologically and biochemically. Sharp et. al. reported higher fibrotic marker levels after repeated doses of CIS [43]. Thus, this regimen was more likely to cause an interstitial fibrosis. Therefore, by a single-dose induction (16 mg/kg of CIS) we could obtain less destructed retinal specimens to assess CIS damage. Polat et. al reported retinal thickness increase and deterioration after single dose CIS administration which caused a thicker RPE in their specimens and presumed that RPE might be the most sensitive region to CIS toxicity [17]. Specimens in this study demonstrated an acidophilic staining with H&E dye which is an early indicator of tissue damage and a slight increase of retinal thickness was observed, as well. Moreover, we performed semi-quantitative analysis to the specimens for a more detailed assessment and observed significant 8-OHdG positive staining particularly in horizontal cells and other retinal neurons. Dulz et al. reported a significant decrease in RNFL thickness values of germ cell cancer patients receiving CIS based chemotherapy [12]. Furthermore, patients' full-field electroretinogram tests suggested mild changes at the cone photoreceptor level with significantly reduced and prolonged cone-b wave latency and a cone-a wave latency [12].

Nitric oxide takes part in basal blood flow of ocular tissues and is produced by three different nitric oxide synthase (NOS) according to the sight [44, 45]. Endothelial NOS (eNOS) is localized in choriocapillaris endothelium and significantly increases in endothelial

dysfunction. It is thought to have a role in ocular hemodynamic abnormalities which lead to various ocular disorders such as ischemic retinopathy and glaucoma [46]**.** In this study, the semi-quantitative analysis with eNOS staining of the specimens yielded larger numbers of stained retinal blood vessels. Kwan et al. reported a case of sudden vision loss due to CIS toxicity who had presented with widespread retinal cotton-wool spots, venous dilation, and microvascular abnormalities [11]. They presumed this toxicity as a vaso-occlusive process and the result of this study, significant eNOS staining of retinal vessels, might be a clear proof for their hypothesis.

As a potent antioxidant, ASTA decreases ROS formation and lipid peroxidation[47]**.** Lipid peroxidation was investigated by detecting tissue MDA levels in all groups and mean MDA level was significantly decreased in the CIS applied group. However, ASTA in either dose 25 mg/kg and 75 mg/kg for 8 days (in sum 200 mg/kg, 600 mg/kg, respectively) had decreased serum mean MDA value approximately to the mean level of the control group. Tong et al. investigated the effect of diosmin against ischemia-reperfusion injury in rat retina[23]. Retinal MDA level was significantly increased in the injured tissues and diosmin decreased MDA levels and was suggested to be protective against this injury [23]. Nakajima et al. administered 400 mg/kg AST in four doses which were estimated to reach at least 100 nM maximal serum concentrations [27]. They reported reduced ROS formation and less in-vitro retinal ganglion cell damage and claimed that even the ASTA concentrations as low as 10 nM could be effective [27]. GSH levels were assessed in both studies which investigate the effect of hesperidin and fish oil against CIS toxicity. Decreased GSH levels in only CIS treated groups and increased GSH levels after administration of antioxidant molecules were reported in these studies [17, 18]. Similarly, serum GSH values were assessed in this study to describe the oxidative damage and reduced GSH levels were observed in the CIS group.

Moreover, both ASTA doses increased GSH levels approximately to the mean level of the control group, which reflects the efficacy of ASTA against oxidation.

The anti-neoplastic effect of CIS was explained by DNA adducts formation, ROS accumulation, lipid peroxidation and mitochondrial stress increase [48]. Even though ASTA shows promise to diminish the toxic effects of CIS treatment, it was not determined in this study whether ASTA also diminishes anti-neoplastic activity. Further studies are necessary to investigate the effect of ASTA on the anti-neoplastic activity of CIS and to define proper doses which protect retinal tissues without sacrificing the main purpose of CIS therapy.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Table 1. IHC Staining Positivity Score.

Grading of the IHC Staining Positivity Score	
0	**None**
1	Mild (less than 5%)
2	Moderate (involvement 5%-25%)
3	Severe (more than %25)

Table 2. Mean ± SD and statistical evaluations of MDA and GSH values.

Groups	MDA (mmol/L)	GSH (mmol/L)
Control	18.13±1.57**	24.40±1.06**
Olive oil	17.77±1.02**	25.14±0.87**
Asta 75mg	14.71±1.02**	28.94±0.61**
Cisplatin	24.01±1.23*	19.19±1.11*
Cisplatin+asta 25mg	16.15±0.66**	26.35±1.30**
Cisplatin+asta 75mg	15.93±0.22**	26.09±1.38**

*p<0.05 versus to control group.
**p<0.05 versus to Cisplatin group.

Tukey Test

Table 3. Semi-qantitative analysis data **(**Median ± SD).

Groups	8-OHdG Positivity score	eNOS positivity score
Control	0.00±0.41	0.00±0.41
Olive oil	0.00±0.41#	0.00±0.41#
Asta 75mg	0.00±0.41#	0.00±0.41#
Cisplatin	1.50±0.55*	1.00±0.52*
Cisplatin+asta 25mg	0.0±0.41#	0.00±0.41#
Cisplatin+asta 75mg	0.00±0.41#	0.00±0.41#

*p<0.05 versus to Control group.
#p<0.05 versus to Cisplatin group.
Kurkal Wallis; Tamhane Test

Table 4. Median ± SD and statistical evaluations of Retinal thickness (µm).

Groups	Retinal thickness (µm)
Control	105.00±9.27
Olive oil	112.57±14.58[#]
Asta 75mg	99.16±4.41[#]
Cisplatin	133.47±6.27[*]
Cisplatin+Asta 25mg	110.85±17.23[#]
Cisplatin+Asta 75mg	102.17±24.50[#]

[*]p<0.05 versus to Control group.
[#]p<0.05 versus to Cisplatin group.
Kruskal Wallis Test

Figure 1. Representative light microscopic photographs from pars optica retinae stained with H&E.

Photosensitive Layer (PL).Outer limiting membrane (OLM), Outer nuclear layer (ONL). Outer plexiform layer (OPL). Inner nuclear layer (INL). Inner plexiform layer (IPL). Ganglion cell layer (GCL). Inner limiting membrane (ILM).

A (x400)-**B**(x400): **Control Group**; Healthy pars optica retinae layer image of the control group. **C** (x400)-**D** (x600): **Olive oil Group**; Pars optica retinae of the regular structure is observed. **E** (x400): **Astaxanthin 75 mg Group**; Pars optica retinae are observed to be in the normal structure. **F**(x600): **Cisplatin Group**; Cisplatin treatment acidophilic staining histological appearance. **G** (x600): **Cisplatin+Astaxanthin 25 mg Group**; Astaxanthin 25 mg treatment no pathology was observed. **H** (x400): **Cisplatin+Astaxanthin 75 mg Group**; Glomerulus in regular structure (gl). Figure demonstrated that the proximal (pt) and distal tubules (dt) are in a stranded structure.

Figure 2. Representative light microscopic photographs from pars optica retinae stained with 8-OHdG**.**

A(x200) Control Group. **B** (x400): **Olive oil Group**; None 8-OHdG immunoreactivity were observed **C**(x600): **Astaxanthin 75 mg Group**; None 8-OHdG expression observed. **D** (x400): **Cisplatin Group**; 8-OHdG positive immunoreactivity were observed (arrow) ($p=0.014$; $p<0.05$). **E** (x600): **Cisplatin+Astaxanthin 25 mg Group**; Astaxanthin 25 mg treatment no immunoreactivity was observed ($p=0.014$; $p<0.05$) **F** (x600): **Cisplatin+Astaxanthin 75 mg Group**; No immunoreactivity was observed ($p=0.01$; $p<0.05$).

Figure 3. Representative light microscopic photographs from pars optica retinae stained with eNOS**.**

A(x200) Control Group. **B** (x400): **Olive oil Group**; None eNOS immunoreactivity were observed. **C**(x600): **Astaxanthin 75 mg Group**; No immunoreactivity observed. **D** (x400): **Cisplatin Group**; Cisplatin treatment eNOS positive immunoreactivity (arrow) were observed ($p=0.025$; $p<0.05$). **E** (x600): **Cisplatin+Astaxanthin 25 mg Group**; Astaxanthin 25 mg treatment no immunoreactivity was observed ($p=0.025$; $p<0.05$). **F** (x600): **Cisplatin+Astaxanthin 75 mg Group**; No immunoreactivity was observed ($p=0.025$; $p<0.05$).